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# Differentiation and adaptation of Natural Killer cells for anti-malarial immunity

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**Running Header**

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**Abstract**

Natural killer cells employ a diverse arsenal of effector mechanisms to target intracellular pathogens. Differentiation of NK cell activation pathways occurs along a continuum from reliance on innate pro-inflammatory cytokines and stress-induced host ligands through to interaction with signals derived from acquired immune responses. Importantly, the degree of functional differentiation of the NK cell lineage influences the magnitude and specificity of interactions with host cells infected with viruses, bacteria, fungi and parasites. Individual humans possess a vast diversity of distinct NK cell clones, each with the capacity to vary along this functional differentiation pathway, which - when combined - results in unique individual responses to different infections. Here we summarise these NK cell differentiation events, review evidence for direct interaction of malaria-infected host cells with NK cells and assess how innate inflammatory signals induced by malaria parasite-associated molecular patterns influence the indirect activation and function of NK cells. Finally, we discuss evidence that anti-malarial immunity develops in parallel with advancing NK differentiation, coincident with a loss of reliance on inflammatory signals, and a refined capacity of NK cells to target malaria parasites more precisely, particularly through antibody-dependent mechanisms.

## Introduction

Natural killer (NK) cells were initially identified as cytotoxic effector cells which recognise cancers in the context of 'missing self' – a process involving the absence or down regulation of self MHC molecules on tumor cells and which was subsequently observed in the recognition of virus-infected host cells [1,2]. NK cells are now recognised as a phenotypically diverse population of innate lymphoid cells expressing a vast array of surface receptors that regulate their function [3]. Whilst much of this heterogeneity relates to individual and population level genetic diversity, environmental factors have a considerable impact on the functional phenotype of NK cells [3-5]. Age and the extent of exposure to infectious agents (particularly persistent viruses) can independently modulate the functional diversification of NK cells [6-9].

Differences in the mode of activation of NK cells impact directly on how these cells are able to integrate immune activating signals resulting from malaria infection. Broadly speaking, less differentiated NK cells have increased reliance on innate inflammation-associated signals for their activation whilst age- and infection-related differentiation promotes integration of NK cell function with adaptive immune signals.

Evidence from experimental malaria infections in mice, humanised mouse models and controlled human malaria infections suggests NK cells are integral to the early immune response and are activated by inflammatory cytokines induced by blood stage infection. However, whilst affording some degree of protection against infection, NK cells can in some circumstances contribute to a pathogenic inflammatory cascade associated with symptomatic disease (reviewed in [10,11]). Conversely, lesser reliance on inflammatory mediators alongside the coordinated maturation of the NK cell compartment and broadening of acquired malaria antigen-specific immunity may enhance protection against both malaria infection and malarial disease [10,11]. By integrating innate cytokine-mediated pro-inflammatory signals with adaptive immune signals induced by malaria parasites themselves, NK cells can act both as sensors of malaria infection and effectors of malaria specific anti-parasite immunity.

Malaria is predominantly a blood-borne disease with growth and replication of asexual parasites and differentiation of transmissible (sexual) stages (gametocytes) occurring in the peripheral circulation, spleen and bone marrow. The interaction of malaria-infected cells with circulating peripheral blood NK cells is thus of potential relevance. However, the initial stage of the infection occurs in the liver where parasite-infected cells may also encounter tissue resident immune cells.

**Human natural killer cell differentiation**

In humans, peripheral blood NK cells are defined according to the expression of CD56 (N-CAM) and the lack of CD3ε chain. Peripheral blood NK cells express variable levels of CD56 – $CD56^{bright}$ cells being a minor population (circa 10% of peripheral blood NK cells) and $CD56^{dim}$ being the majority subset. In the blood, $CD56^{bright}$ NK cells are regarded as the least differentiated subset although a direct precursor relationship with $CD56^{dim}$ NK cells has not been definitively established. A schema representing the current understanding of human blood NK cell phenotypic and functional differentiation is shown in *Figure 1* [12]. Importantly, $CD56^{dim}$ blood NK cells represent a spectrum of NK cell differentiation – a subset of $CD56^{dim}$ cells also express high levels of CD57, a cell surface moiety also associated with advanced T cell differentiation and senescence [13] (*Figure 1*). Interestingly, whilst $CD56^{bright}$ cells have longer telomeres and higher telomerase activity than the highly differentiated $CD56^{dim}CD57^{+}$ subset, irrespective of age, NK cells of all subsets from younger individuals tend to have longer telomeres than NK cells of older individuals, indicating that the differentiation status of NK cells is only partly age-related [14]. Equivalent functional differentiation of NK cells is observed in mice and non-human primates, which may be of relevance to malaria infection models [15].

Progressive differentiation is associated with phenotypic changes which impact the functional propensities of NK cells (*Figure 1*). $CD56^{bright}$ cells are characterised by their high level expression of cytokine receptors – in particular those for IL-12, IL-18, the innate common gamma-chain cytokines, and notably the CD25-CD122 heterodimer which possesses high affinity for interleukin-2 (which enables NK cells to respond to picomolar concentrations of this T cell–derived cytokine) [12,16].

CD56$^{dim}$ NK cells do, however, also express intermediate levels of receptors for interferon alpha and IL-18. Receptors for other cytokines, including IL-2, are upregulated upon activation, consistent with additional regulation of cytokine responsiveness with increasing NK cell differentiation [17-19]. However, the most differentiated NK cells exhibit altered transmembrane and intracellular signalling capacity, leading to reduced overall expression of cytokine receptors and a diminishing reliance on both IL-12 and IL-18-mediated activation (see below).

Variation in expression of receptors for various stress-induced and self-recognition (MHC) ligands also occurs with advancing NK cell differentiation (Reviewed in [12]). CD56$^{bright}$ NK cells express high levels of the natural cytotoxicity receptors (NCR) NKp30 and NKp46, activating receptors of the Ig superfamily that promote interactions of NK cells with accessory cells and have been implicated in interactions with soluble molecules such as complement factor P and viruses, including reoviruses [20-23]. These less differentiated NK cells tend to express the inhibitory c-type lectin-like receptor NKG2A, which, in conjunction with CD94, recognises HLA leader peptides bound to HLA-E (*Figure 1*). As differentiation proceeds towards a CD56$^{dim}$CD57$^{+}$ phenotype, surface expression of NCRs diminishes in parallel with an increase in the frequencies of cells expressing killer immunoglobulin-like receptors (KIR) for MHC class I; at the same time NK cells expressing CD94-NKG2C begin to predominate over those expressing CD94-NKG2A [12] (*Figure 1*). Importantly, whilst only a minor subset of CD56$^{bright}$ NK cells express CD16, the low affinity IgG Fc receptor (FcgRIII), this receptor is expressed on the majority of CD56$^{dim}$ cells with expression levels being highest on CD56$^{dim}$CD57$^{+}$ NK cells [19] (*Figure 1*).

The distribution and diversification of NK cell subsets vary considerably between blood, secondary lymphoid tissues, and inflamed non-lymphoid tissues, which may have consequences for local anti-pathogen responses [15,24]. In the spleen and secondary lymphoid tissues, including tonsils and lymph nodes, NK cells have a less differentiated phenotype, typically CD56$^{bright}$ c-kit$^{-}$ IL-7R$^{-}$ [25,26]. NK cells from these secondary lymphoid tissues produce cytokines such as IFN-$\gamma$ but can be induced by cytokines such as IL-12 and IL-18 to acquire receptors such as KIR (conventionally associated with more differentiated NK cell phenotypes), indicating that these tissue resident NK cells may be

actually be more differentiated than their circulating counterparts. In non-lymphoid tissues, there is significant diversity in NK cell populations[24] including circulating non-resident conventional NK cells (cNK) that are phenotypically similar to $CD56^{dim}$ and $CD56^{bright}$ cells, and tissue resident NK cells (Tr-NK) that vary markedly in phenotype and function between tissues. In the liver, for example, Tr-NK cells express CXCR6 and CD49d but are $CD56^{dim}$ with variable KIR and NKG2C expression. In contrast, uterine NK cells are $CD56^{bright}$ but also bear some hallmarks of conventional $CD56^{dim}$ NK cells including NKG2C and KIR expression [27]. A common feature of Tr-NK cells, however, is a lack of expression of CD16 and CD57, typically found on cNK cells [24].

**Genetic and environmental factors influencing NK cell differentiation and function.**

As with the T and B lymphocyte compartments, there is persuasive evidence that both genetic and environmental factors influence NK cell differentiation and function. Genetic heterogeneity, including population diversity of both HLA and KIR gene alleles, has considerable impact on NK cell function. NK cell responsiveness to cytokines, target cells and antibody complexed to FcR is also intrinsically regulated by a process termed 'education', where increased functional capacity is associated with binding of NK cell receptors to their "cognate" ligands [28,29]. Educating signals include those generated by interaction of inhibitory self-KIR with conventional MHC class I molecules, NKG2A with HLA-E or HLA-G, or invariant NK cell receptors with their relevant ligands (for example the CD2-LFA-3 interaction or CD16 crosslinking by IgG antibodies) [28-30], and can be cumulative. For example, the number of cognate pairs of KIR receptors able to bind to class I MHC molecules determines the overall capacity for NK cell IFN-$\gamma$ production and degranulation [29]. The molecular basis for potentiation of NK cell function by education has recently been reported to involve remodelling of secretory lysosomes, potentially by enhanced $Ca^{2+}$ signalling from acidic cytoplasmic intracellular stores [31]. If so, even though infected red blood cells essentially lack MHC class I molecules, individual genetic variation in NK cell receptors and their licensing ligands will influence the intrinsic capacity of NK cells to mount effector responses to infection, including to blood stage malaria parasites and may, in part, explain reported associations between KIR–HLA ligand pairings and

susceptibility to severe or cerebral malaria.[32,33]. Recent studies on the role of P. vivax-infected reticulocyte MHC class I expression in cytotoxic T lymphocyte responses, however, raise the possibility of direct modulation of NK cell responses under certain conditions [34].

Human cytomegalovirus (HCMV) infection is the most well defined driver of NK cell functional diversification [4,8,35]. Phenotypic and functional differentiation progresses more rapidly in those with HCMV infection, with increased expression of various activating and inhibitory receptors compared to uninfected individuals [4,8,35]. More differentiated cells, including $CD56^{dim}CD57^{+}$ subsets, are increased in frequency in HCMV+ compared to HCMV- individuals [35,36]. Moreover, expansions of cells expressing NKG2C, a differentiation-associated receptor which recognises HCMV-infected host cells in the context of HLA-E binding peptides from the viral UL40 gene, are observed in HCMV+ individuals during natural infection or upon virus reactivation after bone marrow transplantation. [36-38] It is, however, as yet unclear whether NKG2C-HLA-E/UL40 interactions actually drive NK cell differentiation or act to expand already-differentiated cells.

**PLZF – a master regulator of natural killer cell adaptation**

NK cell differentiation and functional diversification is further amplified by variation in components of the signalling cascades associated with cell surface receptors [9]. The transmembrane adaptor proteins CD3ζ and ZAP-70, and the intracellular adaptor protein SAP, define TCR αβ+ and TCR γδ+ T cell lineages, whilst CD19+ B cells are defined by expression of SYK tyrosine kinases and peripheral blood monocytes by the adaptor protein FcεR1γ, SYK and the intracellular adaptor EAT2. At a population level, peripheral blood NK cells express all of these molecules although there are differences in their expression between $CD56^{bright}$ and $CD56^{dim}$ NK cells and, more importantly, between individuals infected with HCMV and uninfected individuals [9]. In HCMV-infected individuals, epigenetic suppression of the promoter of the proteomyeloid zinc finger molecule (PLZF), encoded by the ZBTB16 locus, leads to down-regulation of a cassette of genes expressed in less-differentiated canonical NK cells [9]. When released from the influence of these PLZF-regulated genes, the activation pathways of 'adaptive' NK cells diverge, downregulating intracellular signalling

components associated with myeloid and B cell lineages and freeing up the activity of adaptor proteins typically associated with memory T cell signalling, including CD3ζ, ZAP70 and SAP [7,9]. NK cell adaptation results in two particular functional consequences. Firstly, enhanced signalling through CD3ζ promotes more efficient activation of NK cells via CD16, thereby promoting improved activation and targeting of HCMV-infected target cells via antibody dependent pathways [7,9]. Although adaptive NK cells are predominantly found in HCMV-infected individuals, preferential expansion of FcεR1γ- NK cells by antibody crosslinking of CD16 is observed in response to other pathogens including influenza-infected target cells [7]. A second functional consequence of NK cell adaptation is a loss of expression of cytokine receptors and associated signalling components, resulting in near complete loss of STAT4/p38 MAP kinase activation and inability to produce IFN-γ upon stimulation with these cytokines [9]. Taken together, these major adaptations within the blood NK cell compartment, especially in HCMV-infected individuals, are likely to have important influences on NK cell responses to a range of pathogens, including malaria parasites.

Less differentiated (canonical) NK cells that respond exclusively to cytokines and antibody-responsive 'adaptive' NK cells are polar opposites on a spectrum of activation requirements. Individuals differ in their frequencies of canonical and adaptive NK cells depending on their genetic make-up, age, HCMV infection and exposure to other infections, but, in many individuals, cells at an intermediate stage of differentiation tend to predominate. NK cells at the intermediate stage of differentiation will frequently integrate signals both from innate cytokines and cell surface ligands, including antigen-antibody complexes. NK cells at an intermediate stage of differentiation ($CD56^{dim}CD57^{+}/PLZF^{+}$) retain residual capacity to respond to cytokines and $CD56^{dim}CD57^{-}PLZF^{+}$ NK cells have intermediate levels of both cytokine receptors and FcRs, thereby integrating both of these signals (see Figure 1). Indeed, inflammatory cytokines, in particular IL-18, synergise with antibody-dependent signals to activate $CD56^{dim}CD16^{+}$ NK cells and for tumour cell targeting by adaptive NK cells [18,39]. In summary, the extent to which the NK cells of any given person have differentiated across this spectrum could, at one extreme, mean that NK cells act primarily as sensors of malaria-induced pro-inflammatory signals and contribute to the malaria induced

inflammatory cascade; at the other end of this spectrum, NK cells mediate very effective antibody-dependent cellular cytotoxicity (ADCC), thereby contributing to protective immunity whilst limiting exacerbation of inflammatory processes. In practice, this implies that NK cell responses to malaria may vary between individuals and over an individual's life course, with very different implications for their role in protection or pathogenesis.

**Malaria parasite induction of the inflammatory cascade and NK cell activating cytokines**

Numerous studies of malaria infection in both animal models and humans have described the induction of inflammatory and anti-inflammatory cytokines as being crucial to determining the severity of disease and eventual outcomes. Erythrocytic stages of the parasite are most strongly associated with the induction of inflammatory cytokines, including the NK cell-activating cytokines IL-12, IL-18 and type 1 interferons.

Over the past decade, an increasing number of malarial pathogen- or danger-associated molecular patterns (PAMPs and DAMPs) have been identified alongside their myeloid accessory cell receptors; these interactions drive the production of a diverse array of pro- and anti-inflammatory cytokines, including those modulating NK cell activity. Malaria-derived DAMPs and PAMPs include nucleic acids and by-products of intraerythrocytic growth and replication including GPI anchor domains from malarial proteins and modified haemozoin (reviewed in [40]). Binding of these molecules to an array of innate recognition receptors, including TLR9, TLR7 and MyD88, has been implicated in the induction of IL-18, IL-12 and IFN-$\alpha$ in human and murine myeloid cells [41-45]. For example, *P. falciparum*-infected red blood cells (iRBC) have been shown to induce IL-18 in murine monocytes *in vitro* and stimulation of murine bone marrow-derived myeloid dendritic cells from TLR9 or MyD88 knockout mice revealed an essential role for these pathways in the IL-12 response to *P. falciparum* iRBC and to parasite DNA-protein or DNA-carbohydrate polymer complexes [41,45]. Interestingly, in murine *P. yoelii* infection, production of IL-18 is MyD88-dependent but TLR9 independent, and serum IL-12 increased in TLR9-/- mice coincident with a downregulation of IL-10 production [42], whilst production of IFN-$\alpha$ requires STING-mediated detection of parasites by macrophages [44]. These

data are therefore indicative of a complex interaction between inflammatory and anti-inflammatory cytokines which could impact the overall NK cell response. Significantly, the AT-rich stem loops prevalent in *P. falciparum* DNA have been implicated in TLR-9 independent recognition by the STING, TBK1 and IRF3-IRF-7 pathways [46] and TLR7-MyD88-mediated recognition of *P. yoelii* DNA is implicated in the activation of plasmacytoid DCs [44].

Inflammasome-mediated induction of inflammatory cytokines, in particular IL-1β and IL-18, has also been described during malaria infections: *P. berghei* genomic DNA complexed to normally inert malarial hemozoin activates bone marrow-derived murine macrophages via TLR-9, providing priming and activation signals for NLRP3/AIM2 inflammasomes [47,48] and circulating immune complexes containing *P. falciparum* and *P. vivax* DNA can also induce inflammasome assembly, caspase I induction and increased production of IL-1β and IL-18 (RNA) in human monocytes. [49].

In summary, the induction of cytokines with documented potential to activate NK cells is evident in both human and murine malaria infections and involves the co-operation of a number of distinct molecular patterns and signalling pathways in diverse myeloid cell populations.

**Linking inflammatory cytokines to *P. falciparum*-induced NK cell activation**

*In vitro* studies with human cells and *in vivo* studies with animal models have all demonstrated that NK cells are dependent upon accessory cells and inflammatory cytokines to respond to malaria parasite iRBC. Neutralisation of IL-12 and IL-18 abrogated the NK cell IFN-γ response to *P. falciparum* iRBC and schizont lysates [50]. Subsequent studies have demonstrated a requirement for contact between primary NK cells and accessory cells implying that, in addition to accessory cell-derived cytokines, cell contact is necessary for full activation (*Figure 2*). An array of myeloid and T cell-derived NK cell-activating cytokines are induced by *P. falciparum* iRBC stimulation of human PBMC, with upregulation of IL-12, IL-15 and IL-18 RNA and IL-2 and IL-12 proteins detected *in vitro* [51]. In addition to IFN-γ production, many studies have also demonstrated the induction of IL-2Rα (CD25) on the NK cell surface indicating that IL-2 from malaria specific CD4+ T cells may synergise

with innate cytokines in NK cell responses to iRBC [52,53]. Indeed, the contribution of both accessory cell- and CD4+ T cell-derived cytokines to the NK cell response was subsequently confirmed by IL-12, IL-18 and IL-2 neutralisation, anti-IFN-αβR2 and MHC class II blockade, and CD4+ T cell depletion from PBMC cultures prior to activation with iRBC [54]. Interestingly, in this system IFN-γ production from NK cells preceded that of CD4+ T cells, consistent with a dominant contribution of NK cells to the early immune response to iRBC.

One feature of these *in vitro* systems is potent responses by $CD56^{bright}(KIR^{-})$ NK cells and $CD56^{dim}$ NK cells expressing CD94-NKG2A, which is consistent with the activation of less-differentiated NK cells as might be expected for cytokine-driven responses determined by the constitutive expression or upregulation of the appropriate cytokine receptors [51,53,55]. Contact-dependent signals for NK cell responses to iRBC seem to be largely restricted to the level of accessory cells where LFA-1 on NK cells may promote association with cytokine-producing accessory cells via interaction with ICAM-1 [51,52] (*Figure 2*). By contrast, the need for direct contact between NK cells and iRBC is much less clear.

Although NK cells have been shown to form stable conjugates with iRBC [52,53] and purified NK cells respond to *P. falciparum* iRBC with a gene expression signature that is very different to that induced simply by exposing them to IL-12 + IL-18 (suggesting that additional activation signals may be provided by conjugate formation), [56] there is, as yet, no convincing evidence that direct contact with iRBC is essential for NK activation, and no activating (or inhibitory) receptor-ligand interactions have been defined. Early reports [57,58] suggested a role for interaction between the Duffy binding like domain DBL-1α of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) and the natural cytotoxicity receptor NKp30 (and to a lesser extent NKp46) but have not been confirmed, although an association has been observed between polymorphisms in the *NCR3* gene encoding NKp30 and the frequency of mild malaria episodes [57,58]. Another study indicated that chondroitin sulfate modified proteins on human NK cell lines can mediating binding to PfEMP-1 on the iRBC surface, but there was no evidence that this led to NK cell activation [52]. More recently, a role has been suggested for *P. falciparum*-derived microvesicles containing long non-coding RNAs in direct activation of NK cells

via the cytosolic sensor MDA5 [59]. There is also evidence that iRBC can inhibit NK cell activation via the interaction of certain *P. falciparum* RIFIN proteins with LILRB1 (also known as LIR1, LAIR1 or CD85j), a lymphocyte expressed inhibitory receptor of the immunoglobulin superfamily, [60] but the relevance of this – or of any other form of direct contact between NK cells and iRBC – for NK cell activation or immunity to malaria is currently unclear.

**Cytokine-induced NK cell responses in murine malaria**

The inevitable limitations associated with experimental studies of human malaria mean that many of our insights arise initially in animal models of malaria, especially rodent models. Murine malaria models currently provide evidence for both beneficial and harmful contributions of NK cells - either controlling parasite burden or contributing to pathology. It has been recognised for some time that the genetic background of the host and the parasite species are important considerations influencing NK cell activity in these models [61,62]. Nonetheless, these models offer the potential for the mechanistic dissection of the cytokine cascade and NK cell activation, and their contribution to the anti-parasite immune response. Cytokine-activated NK cells do appear to be involved in protection in acute *P. chabaudi* infection: IL-12 restores NK cell function and control of *P. chabaudi* AS parasitaemia in susceptible A/J mice whereas deletion of the IL-12 gene results in severely impaired IFN-γ production, increased peak parasitaemia and delayed resolution of infection in normally resistant C57BL/6 mice [63,64]. Delayed parasite clearance also occurs in IL-15-/- C57BL6 mice and is associated with reduced NK cell and DC function [65]. As described above for accessory cell dependence in human NK cell responses, DC-NK crosstalk is critical for NK cell IFN-γ dependent immunity to *P. chabaudi* in C57BL/6 mice [66]. Further studies of *P. chabaudi* infection demonstrate waves of NK cell activation and proliferation in the blood and spleen with NK cell numbers peaking at the time of peak parasitaemia [67,68]. IL-18-dependent induction of the high affinity IL-2R expression on NK cells and their production of IFN-γ occurred earlier and with higher magnitude after infection of C57/BL6 mice with non-lethal *P.yoelii* 17XL compared to the lethal *P. yoelii* M strain, consistent with a protective role for cross-talk between IL-2-producing T cells and NK cells in this model [69].

Furthermore, control of *P.yoelii* 17XL parasitaemia and survival were both impaired in CD36-deficient C57BL/6 mice and this was associated with decreased production of IL-12, IL-18 and IL-1β, and subsequent decreased NK cell production of IFN-γ and TNF-α, consistent with a role for interactions between NK cells and accessory cells in immunity [70].

If *P. chabaudi* and *P. yoelii* provide evidence for a protective role of cytokine-activated NK cells in controlling parasite replication and preventing death from hyperparasitaemia, *P. berghei* ANKA infection in C57BL/6 mice provides a model for NK cells to contribute to severe disease with reciprocal DC activation and IL-12-dependent NK cell responses being associated with severe inflammation, CD8+ T cell activation and onset of experimental cerebral malaria [71]. Interestingly BALB/c mice, which are normally resistant to *P. berghei* ANKA, become susceptible to experimental cerebral malaria when backcrossed against the C57BL/6 NKC locus, implying a role for functional NK cell receptor involvement in susceptibility to disease [72]. Other studies suggest a role for NK cells in liver injury caused by *P. berghei* NK65 in C57BL/6 mice [73].

The timing and magnitude of the NK cell response may however alter the course of disease in these infections. For example, expansion of the NK cell population by Flt3 ligand treatment of C57BL/6 mice facilitates control of *P. berghei* ANKA parasitaemia and prevents the onset of experimental cerebral malaria via MyD88 and IFN-γ dependent pathways [74]. More recently, a role for NK cell regulatory function in prevention of experimental cerebral malaria was demonstrated in *P.berghei* ANKA-infected C57/BL6 mice in which a therapeutic IL-15 complex induced IL-10-producing NK cells [75].

Further evidence for a role for NK cells during malaria infection comes from humanised mouse models of *P. falciparum*. Depletion of NK cells and macrophages facilitates sporozoite infection of hepatocytes and growth of liver stage *P. falciparum* in humanised mice, suggesting a role for these cells in controlling pre-erythrocytic infection by an as yet undefined mechanism [76]. Similarly, *P. falciparum* parasitaemia induces IL-12 and IFN-γ production one week post infection even in NOD/SCID mice in which NK cells are the only plausible source of this IFN-γ [77]. Finally, depletion of

NK cells from immune cell optimised humanised (RICH) mice demonstrates a role for contact-dependent NK cell IFN-γ production in control of parasitaemia [78].

**NK cell activation during natural malaria infections**

Many studies associate the production of pro-inflammatory cytokines, including those which can activate NK cells, with severe malarial disease, raising the question of whether NK cell activation by these pathways necessarily contributes to protective immunity in susceptible human and animal hosts [79]. In reality, given the heterogeneity in both functional differentiation of NK cells and inflammatory responses within the affected population, it is likely that the answer lies in both the concentrations of pro- and anti-inflammatory mediators being produced and the frequencies of different NK cell subsets responding to these mediators in a given individual.

NK cell-activating, NK cell-derived and NK cell-modulating cytokines are all associated with the severity of malarial disease. For example, ratios of pro-inflammatory IL-12, IFN-γ and TNF-α to anti-inflammatory TGF-β and IL-10 in iRBC-stimulated whole blood were associated with protection against parasitaemia, clinical malaria and anaemia in a study of Ghanaian children [80] and reduced concentrations of plasma TGF-β and IL-12 were associated with severe malaria and cerebral malaria in Thai adults and Tanzanian children [81]. Interestingly, in the latter study, plasma IL-18 was found at higher concentrations in people with uncomplicated malaria compared to uninfected controls but IL-18 concentrations declined with increasing disease severity, suggesting that inflammatory mediators could be involved in the control of parasitaemia, thereby preventing disease [81]. By contrast, studies in Mali and Malawi suggest a direct association between increasing plasma IL-12 concentrations and severe disease or cerebral malaria [82,83], perhaps revealing the importance of also considering the role of potentially disease-modifying concentrations of anti-inflammatory cytokines. NK cell activation (as defined by CD69 expression) was also elevated in cerebral malaria in a related study [83]. Whilst suggestive of a role for NK cell-activating and NK cell-modulating cytokines in determining the outcome of malaria infections, the currently available data are far from definitive [80,82,84,85]. Firstly, few if any studies have sought to directly correlate plasma cytokine concentrations or *in vitro*

stimulated cytokine production with NK cell activation and function. Secondly, the vast majority of these studies are cross-sectional in design and therefore cannot infer causality from any of the associations detected. To fully understand the interplay between inflammation and NK cell responses and the implications of these for control of malaria infections, much more comprehensive, longitudinal studies are needed – including of controlled experimental human malaria infections – in which cellular and cytokine responses are followed over time, ideally including pre-infection and post-treatment time points.



**Evidence for early NK cell activation during controlled human malaria infections**

As discussed above, controlled human malaria infections (CHMI) provide an opportunity for longitudinal studies of infections of known magnitude and duration, and the recent establishment of CHMI protocols in a number of laboratories - in endemic as well as non endemic areas - is providing a rich source of data on a variety of immune cells, including NK cells. Validating earlier *in vitro* studies [54], infection of malaria naïve volunteers via the bites of *P. falciparum* sporozoite-infected mosquitoes, followed by drug cure at the onset of patent parasitaemia, increased the frequencies of both $CD56^{bright}$ and $CD56^{dim}$ NK cells producing IFN-γ, due in part to the infection-induced differentiation of IL-2-producing malaria-specific memory T cells which potentiate innate NK cells responses [86,87]. In a rather different study, using a blood stage inoculum to initiate infection, frequencies of NK cells and type 1 innate lymphoid cells (ILC-1) decreased in the blood as infection progressed but rapidly returned to pre-infection levels after treatment, suggesting that these cells may have been activated to express adhesion molecules, leading to transient sequestration in peripheral tissues [88]. Similar observations of transient lymphocyte sequestration have long been reported in children naturally infected with malaria [89]. Whilst these data are indicative of generalised lymphocyte activation during blood stage malaria infection, this tissue sequestration makes it very difficult to study malaria-

reactive lymphocytes during acute infection since activated - and therefore putatively protective - cells are absent from the peripheral leukocyte population that can be sampled. This may, in part, explain why NK cell gene expression signatures were negatively correlated with and predicted protection following CHMI of individuals vaccinated with the RTS,S malaria vaccine [90]. On the other hand, sporozoite-induced CHMI in a group of malaria-exposed but non-immune Tanzanian adults markedly reduced the proportion of circulating NK cells (and, at high doses of inoculating sporozoites, increased the frequencies of CD8+ mucosal associated invariant T cells, or MAIT cells) [91]. These changes persisted for several months after drug clearance of parasites, suggesting that in these individuals transient activation and sequestration may not explain lymphocyte dynamics [91]. As discussed earlier, more comprehensive CHMI studies are needed to better understand the contribution of NK cells and other lymphocytes to malarial immunity, including *ex vivo* analysis of peripheral blood cell phenotype and function during acute disease and restimulation/recall response analysis after treatment and restoration of homeostasis.

**NK cells as adaptive effectors of acquired immunity to malaria.**

There is abundant evidence from both vaccination and infection studies that NK cell function could be enhanced by T cell derived IL-2 [54,92-95] and by specific antibodies [96-99] , each of which are hallmarks of an adaptive immune response. As discussed above, CD25 - a component of the high affinity receptor for IL-2 - is constitutively expressed on less differentiated human CD56$^{bright}$ NK cells and is induced by activation on more differentiated CD56$^{dim}$ NK cell subsets; CD25 is also a marker of activation in murine NK cells [17].

Consistent with the ability of NK cells to integrate both innate and adaptive immune signals, IL-18 plays a critical role in the induction of CD25 on NK cells in a number of infections, including murine CMV (MCMV) and murine malaria [17,69]. Importantly, expression of IL-18R is maintained on the majority of human NK cells irrespective of their differentiation state, allowing them to continue to respond to IL-18 [19]. This is in sharp contrast to IL-12, where expression of the IL-12R β2 chain declines significantly and progressively as NK cells differentiate, making them less responsive or

non-responsive to IL-12, which can otherwise potently synergise with IL-18 to enhance CD25 expression in less differentiated human NK cells [18,19]. However, whether more highly differentiated NK cells, including both CD56$^{dim}$CD57+NKG2C+ and adaptive CD56$^{dim}$FcεR1γ-/PLZF- subsets, remain sensitive to IL-2 is not yet entirely clear, although CD57+(NKG2C+/-) NK cells do have lower intrinsic proliferative capacity [93,100] and often respond poorly to T cell-activating recall antigens [18,19,93]. If the reliance of these NK cell subsets on IL-2 is genuinely restricted, alternative factors such as IL-15 may be required for their maintenance [18].

Taking all the evidence together, it seems that as NK cells differentiate they progressively lose reliance on both innate cytokines and T cell derived IL-2 for their activation, and thus tend to produce less IFN-γ. Highly differentiated 'adaptive' NK cells ultimately become reliant on direct contact with target cells or immune complexes for their activation and thus on cytotoxic mechanisms of action; in the case of malaria infection this translates into an almost exclusive reliance on activation via CD16 and antigen-IgG immune complexes and thus on ADCC as the primary NK cell-mediated effector mechanism.

Evidence from *in vitro* studies and from longitudinal studies of human malaria infection supports the notion of a switch from innate cytokine/T cell-mediated NK cell activation towards protection dependent on malaria-specific antibody-dependent NK cell responses [10]. Early observations of antibody-dependent NK cell activation in response to iRBC [101] have been confirmed by more recent studies providing convincing evidence that antibodies to *P. falciparum* PfEMP-1 and RIFINs (both of which are expressed at the surface of iRBC) can inhibit parasite replication and kill malaria-infected erythrocytes *in vitro* in the presence of NK cells derived from malaria naïve individuals [102]. Of note, human anti-malarial antibodies belong almost exclusively to the IgG1 and IgG3 subclasses (the Fc regions of which preferentially bind CD16) and have repeatedly been associated with protective immunity to malaria [103-106]. As ADCC is preferentially mediated by more differentiated CD56$^{dim}$CD57+NKG2C+ and CD56$^{dim}$FcεR1γ-/PLZF- NK cells that accumulate with increasing age, NK cell maturation in combination with gradual acquisition of antibodies to a broad repertoire of PfEMP-1 and RIFIN serotypes may contribute to the well documented phenomenon of age- and

exposure-related acquisition of effective anti-malarial immunity. The almost universal exposure to HCMV in sub-Saharan African populations, concomitant exposure to other pro-inflammatory infections (potentially including malaria itself) [7,107-109] and the consequent rapid accumulation of CD57+ NKG2C+ NK cells [36] may accelerate this process. In support of this hypothesis, a recent study in Malian children and young adults reported that peripheral blood frequencies of PLZF- adaptive NK cells were positively associated with ADCC against *P. falciparum*-infected erythrocytes and inversely correlated with parasite burden and probability of infection in the subsequent malaria season [110].

**Cytokine-dependent NK activation after malaria vaccination**

The role of NK cells as effector cells of vaccine-induced protection has been proposed by ourselves and others (reviewed in [111]). With regard to malaria, enhanced IFN-γ production by NK cells was demonstrated after RTS,S vaccination and was correlated with vaccine antigen-specific IL-2 production [92]. However, as described above, CHMI of RTS,S vaccinated individuals suggested an inverse correlation between NK associated gene signatures and protection [90], although this may simply reflect tissue sequestration of activated NK cells during active infection. On a more positive note, liver IL-12 and NK cell signatures were associated with protection in a *P. chabaudi* vaccination and challenge study [112,113] and a *P. vivax* vaccination study reported increased NK cell frequencies after vaccination [114].

**Concluding remarks: the multifaceted role of NK cells in malaria**

Observations from natural human infection, *in vitro* systems and animal models broadly support the notion that NK cell responses to malaria in naïve or non-immune individuals are largely driven by innate, pro-inflammatory cytokines induced principally by erythrocytic stages of *Plasmodium spp*. We propose, therefore, that in malaria endemic populations, the gradual acquisition of acquired immune responses (both memory T cells and specific antibodies) is mirrored by rapid differentiation

of NK cells and accumulation of adaptive NK cells with potent ADCC capability; the transition from dependence on largely innate to largely adaptive NK cell-activating signals is smoothed by the ability of intermediate differentiation stages of NK cells to interpolate both sets of signals, including T cell-derived IL-2 . *Figure 3* shows a proposed model for the gradual evolution of the NK cell effector response to malaria-infected erythrocytes over the life course of an individual. In this model, age and exposure to HCMV and other pro-inflammatory infections (including malaria) leads to gradual differentiation of the NK cell population from being reliant for its activation on malaria-induced inflammatory cytokines towards increasing reliance on malaria antigen-antibody immune complexes. At the same time, acquisition of immune regulatory mechanisms that moderate the inflammatory cytokine response to malaria and the increasing diversity of the anti-malarial antibody response enable NK cells to mediate very effective ADCC responses and to clear the infection with minimal inflammation. If so, it should be possible to track this 'evolution' of the NK cell-mediated anti-malarial immune response - both phenotypically and functionally – in malaria-exposed individuals to reveal informative, composite correlates of protection against malaria infection and disease. At the same time, the potential for anti-inflammatory cytokines (in particular IL-10 and TGF-β) to moderate the function of less differentiated, cytokine-responsive and cytokine-producing NK cells should be explored. In addition, given recent evidence from murine infection and vaccination models that NK cells may inhibit affinity maturation of immunoglobulins by negatively regulating somatic hypermutation in germinal centres [115,116], it may be of value to correlate NK cell phenotype and function with the affinity maturation of the anti-malarial antibody response.

There is abundant evidence that host genetic diversity contributes to resistance/susceptibility to malaria and to variation in individual and population level NK cell responses to malaria parasites. The interaction between polymorphic inhibitory KIR and HLA class I molecules helps to determine the functional competence of NK cells [29] and a number of studies have suggested an association between KIR genotype and the severity of malaria disease [32,33,117,118]. At the same time, polymorphisms in genes encoding cytokines and cytokine receptors [119-121] and immunoglobulin Fc receptors including CD16 and CD32 [122-124] may affect the avidity of these interactions, the activation of NK cells and – directly or indirectly – the outcome of malaria infections. Traits that influence NK

cell differentiation may also have functional consequences: for example a deletion variant of *NKG2C*, present at high allele frequency in some African populations, is associated with delayed NK cell differentiation [36] and may have implications for the generation of effective ADCC responses to malaria.

Lastly, differences in the functional and phenotypic characteristics of NK cells in the peripheral blood and tissues may merit consideration. The relative over-representation of less differentiated NK cells in secondary lymphoid organs and other tissues compared to peripheral blood, where highly differentiated CD16+ and 'adaptive' NK cell types tend to accumulate, may mean that the entire NK cell pool remains rather more diverse than might be apparent from the circulating NK cell population, with important consequences for immunity to malaria and other infections. As our understanding of the dynamic spectrum of NK cell differentiation and responsiveness during infection increases, we may likewise better understand the role of innate lymphocytes during anti-malarial immunity and in the evolution of natural protection against disease.

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## Figure Legends

**Figure 1.** Human NK cell differentiation and adaptation. CD56$^{bright}$ NK cells express high levels of activating NCRs and cytokine receptors. As NK cells begin to differentiate, they begin to downregulate cytokine receptors and NCRs and to express CD16 and KIR. Highly differentiated NK cells express CD57, high levels of KIR and LIR-1, and may express CD94/NKG2C+ at high frequency. Adaptive NK cells, which are assumed to differentiate from CD56$^{dim}$ (CD57- or CD57+) NK cells lose expression of the FcεR1γ adaptor protein and the transcriptional regulator PLZF.

**Figure 2.** Activation of NK cells by malaria parasites. Myeloid cells (monocytes, macrophages and myeloid DCs) recognise soluble components of blood stage *Plasmodium spp* and phagocytose infected erythrocytes and extracellular parasites, leading to triggering of PRRs (including TLR4 and TLR9) and release of NK cell-activating pro-inflammatory cytokines. Myeloid cells also provide accessory signals to NK cells via cell surface receptors including adhesion molecules, leading to activation of NK cells and their secretion of IFN-γ. Plasmacytoid DC can additionally recognise parasite DNA (complexed to hemozoin or other parasite proteins) via TLR9-independent pathways, leading to type 1 interferon production and further activation of NK cells. Adapted from Newman and Riley [125] ; symbols as in Figure 1.

**Figure 3.** Model for the co-evolution of adaptive NK cells and anti-malarial immunity. With increasing age and repeated malaria infections, children in malaria endemic areas gradually acquire adaptive (T cell and antibody) immunity. At the same time, their NK cells gradually differentiate from cytokine-activated/cytokine-producing cells to become specialised for ADCC. We propose, therefore that early in life (**panel A**) when antibodies are lacking, the NK cell response is driven by malaria-induced inflammation and NK cells secrete IFN-γ which enhances the phagocytic clearance of infected erythrocytes but may also contribute to inflammatory disease. With increasing age and malaria exposure (**panel B**), the acquisition of IL-2-producing memory T cells and anti-malarial antibodies

may enhance both cytokine-driven NK cell effector mechanisms and ADCC. In later life (**panel C**), an increasing ability to modulate (or actively regulate) the inflammatory response to malaria, combined with maturation of the anti-malarial antibody response and accumulation of “adaptive” NK cells, results in control of infection via very effective ADCC with minimal inflammation. Cells and symbols as shown in legend to Figures 1 and 2.

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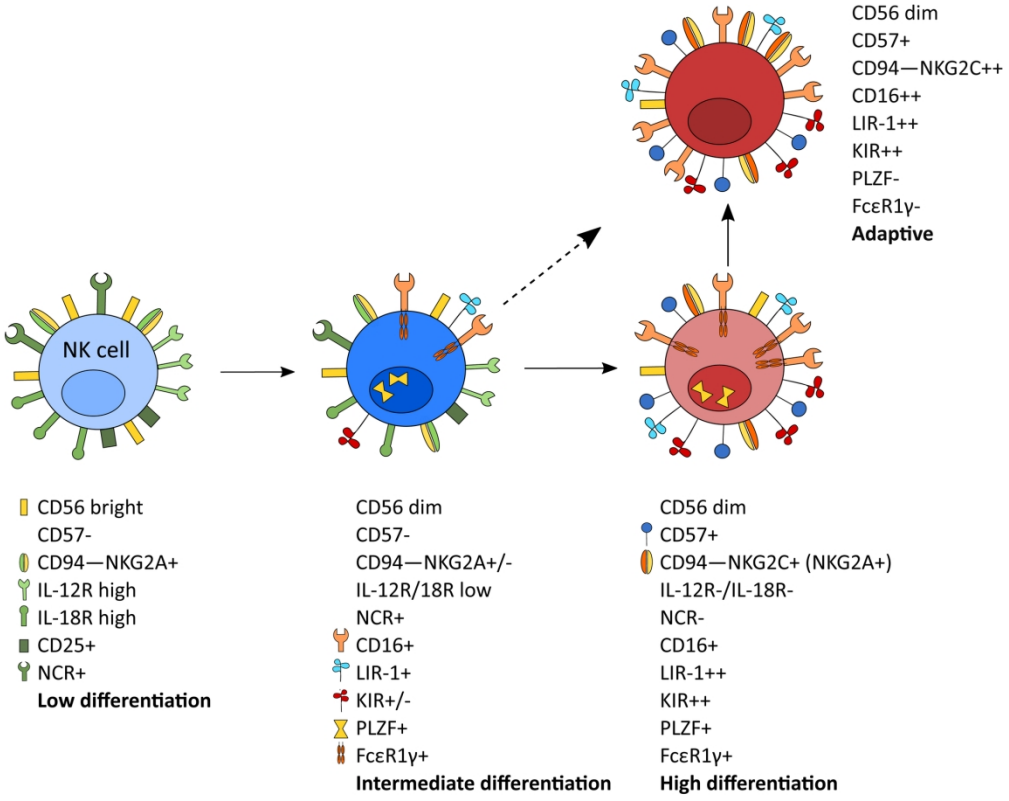


Figure 1

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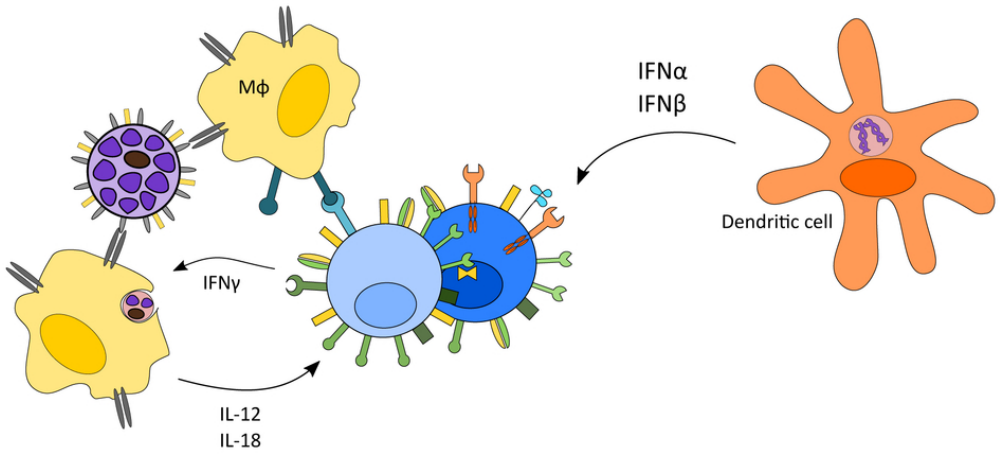


Figure 2

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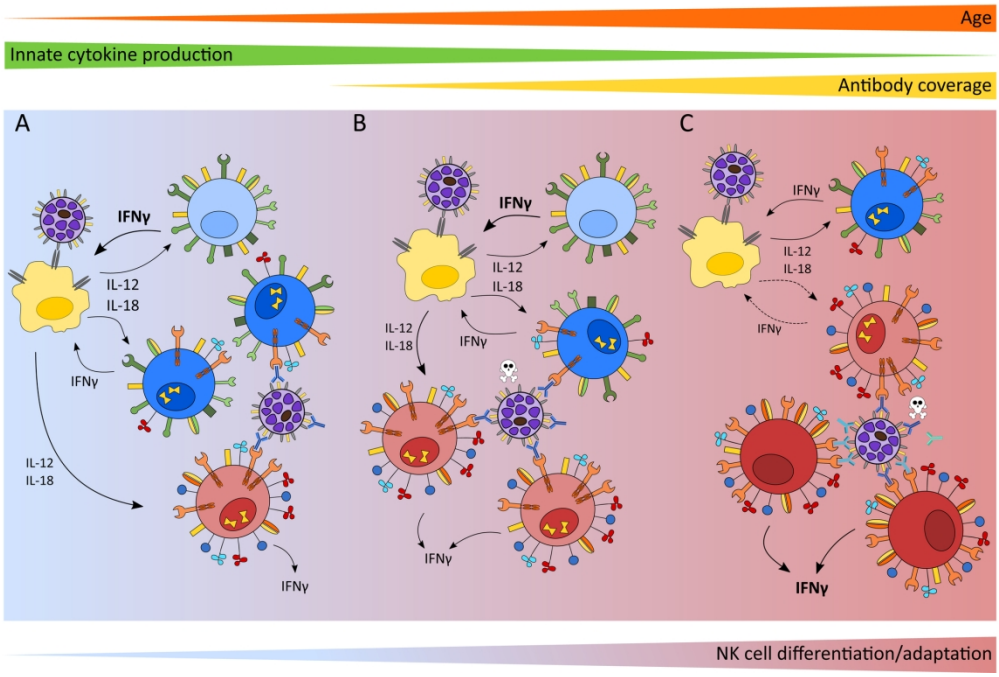


Figure 3

180x120mm (300 x 300 DPI)